Pergamon

PII: S0031-9422(97)00964-3

Phytochemistry, Vol. 49, No. 3, pp. 657–666, 1998
© 1998 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0031-9422/98/$—see front matter

AMYLASES SYNTHESIS IN SCUTELLUM AND ALEURONE LAYER OF MAIZE SEEDS

KOLLI V. SUBBARAO,† RUPALI DATTA and RAMESHWAR SHARMA*

School of Life Sciences, University of Hyderabad, Hyderabad 500 046 India; † ProAgro Seed Company Ltd., Tolichowki, Hyderabad, India

(*Received in revised form* 13 *October* 1997)

Key Word Index—*Zea mays*; Gramineae; maize; amylase synthesis; secretion; α-amylase; β-amylase; aleurone layer; scutellum.

Abstract—The endosperm of germinating maize seeds contains four isozymes of α-amylases (α-amylase-1 to -4) and one isozyme of β-amylase. The α-amylases were purified by affinity chromatography on amylose and separated by DEAE-cellulose chromatography, into two groups, namely α-amylases-1,2 and α-amylases-3,4; and β-amylase was purified by precipitation as a glycogen-enzyme complex. The molecular weight of α-amylases-1 and -2 was 46 kD, α-amylases-3 was 44.5 kD and of α-amylases-4 was 47.5 kD. The molecular weight of β-amylase was 56 kD. During seed germination increase in amylolytic activity in endosperm was mainly contributed by secretion of α-amylases from adjoining aleurone layer and scutellum. The synthesis and secretion of α-amylases was first initiated in the scutellum followed by aleurone layers. Exogenous Ca^{2+} stimulated synthesis of α-amylases in both eleurone layer and scutellum. In contrast, though scutellum and aleurone layer synthesized β-amylase but it was not secreted to the medium. These results suggest that during the early germination period, α-amylases secreted from scutellum mobilizes starch. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

In cereals seeds, the germinating embryo obtains the necessary energy by the degradation of carbohydrates present in the endosperm. The molecular and cellular biology associated with the endosperm mobilization has been studied in great detail in several cereal species [1]. In barley, synthesis of the enzymes involved in the mobilization of the endosperm after the imbibition of seeds is highly regulated. The aleurone layer here acts as a site for hormone-controlled induction of several enzymes [2].

α-Amylases constitute a major portion of the secretory proteins released from the aleurone layer to the endosperm for starch mobilization. The hormonal and developmental regulation of α-amylase activity during barley seed germination has been extensively studied [2, 3]. The induction of α-amylase gene expression in barley is promoted by gibberellic acid and is repressed by ABA [3]; it also depends on the availability of calcium [4]. Cloning of α-amylase genes from barley indicated that these genes are clustered in two multi-gene families located on chromosomes 1 and 6, encoding α-amylase proteins of low and high pI respectively [5]. Additionally, the members of these gene families are differentially regulated by calcium and hormones [6].

There are relatively few reports about the expression and regulation of α-amylases in maize and other cereals belonging to the Panicoideae subfamily of Gramineae. Availability of mutants defective in starch synthesis in maize has been useful in understanding the role and the regulation of the enzymes involved in starch biosynthesis in developing endosperm of maize seeds [7]. In maize, α-amylase synthesis was independent of hormonal regulation during seed development [8] and seed germination [9], implying the absence of a major role of hormones in α-amylase induction. In germinating maize seeds, amylase activity comprises of several isoforms of amylases, the appearance of which are temporally regulated [10, 11], and could be modulated by gibberellic acid and ABA. This amylolytic activity was contributed by both the aleurone layer and the scutellum, and the secretion of amylases was calcium independent [12]. The β-amylase level also increased during maize seed germination [13].

In the present study, we purified two groups of α-amylases; α-amylase-1/2, α-amylase-3/4 and a β-amy-

* Author to whom correspondence should be addressed. Tel: +91-40-3010514; Fax: +91-40-3010120; E-mail: rpssl@uohyd.ernet.in

lase from maize seeds. Specific polyclonal antibodies obtained against the above proteins were used to study this *in vivo* synthesis and secretion from the scutellum and the aleurone layer of maize seeds.

RESULTS AND DISCUSSION

Amylase purification

Amylases of 6-day-old maize endosperm were resolved into 5 isoforms on non-denaturing PAGE (Fig. 1C, lane 4). On the basis of their substrate specificity, four of these isoforms were classified as α-amylase and one as β-amylase; as α-amylases can degrade β-limit dextrin but not β-amylase, whereas both enzymes can degrade amylose. α-Amylase isoforms were numbered 1 to 4 based on their mobility on non-denaturing PAGE, with the α-amylase isoform on the top of the gel being designated as α-amylase-1. β-Amylase was the fastest moving isoenzyme, closest to the cathode end of the gel (Fig. 1C. lane 4). A similar pattern for maize amylases was also reported by Wang *et al.* [13].

Separating α- and β-amylases and the different isoforms of amylases is difficult. Rice α- and β-amylases could be separated by ion-exchange chromatography and chromatofocusing [14]; maize α-amylases were separated from β-amylase by chromatofocusing [11]. Selective binding of α-amylases to their substrates or inhibitors may be used to separate them from β-amylase (e.g. glycogen precipitation of α-amylase [15] and cycloheptaamylose chromatography [16]).

In this study, potato amylose affinity chromatography separated most of the α-amylase activity from β-amylase and also led to the purification of α-amylases to near homogeneity (Table 1). While α-amylases were selectivity adsorbed onto amylose, β-amylase was recovered in the unbound fraction. However, the amylose column did not completely retain α-amylase activity, and some α-amylase activity was also present in the unbound fraction containing β-amylase.

The dextrin eluate of amylose column contained all the four isoforms of α-amylase which showed four corresponding bands on non-denaturing PAGE (Fig. 1A, lane 1) and three protein bands on SDS-PAGE (Fig. 1B, lane 1). On DEAE-cellulose chromatography (Tris-HCl, pH 8.5) these isoforms were separated into two distinct peaks; peak-I and peak-II; containing α-amylase-1/2 (Fig. 1A, lane 2) and α-amylase-3/4 (Fig. 1A, lane 3) isoforms respectively. On SDS-PAGE, α-amylase-1/2 migrated as a single band (Fig. 1B, lane 2), whereas α-amylase-3/4 showed two bands (Fig. 1B, lane 3).

α-Amylases contained in peak II were resolved by Sephadex G-100 chromatography. α-Amylase-4 isoform was eluted predominantly in the early fractions

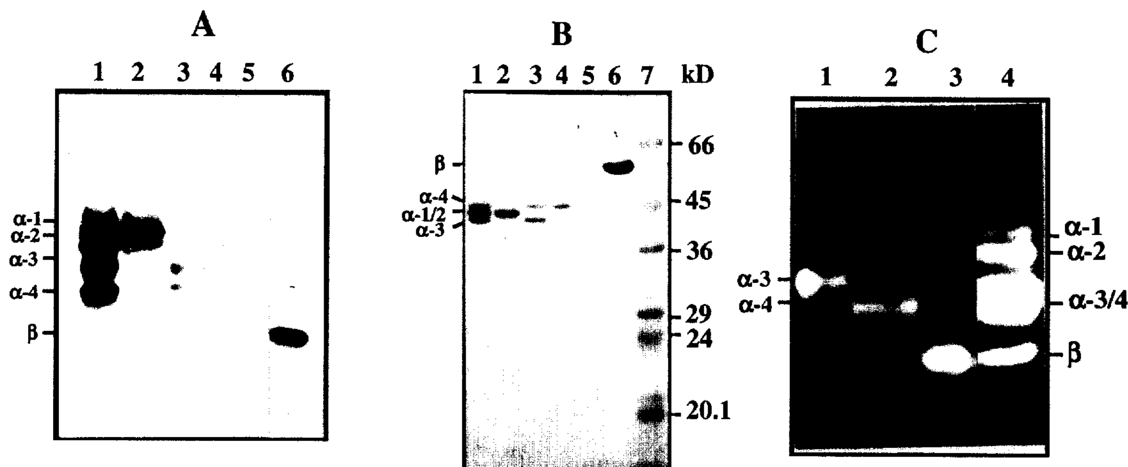

Fig. 1. Analysis of amylases by electrophoresis during different steps of purification. The symbols α- and β- on figures indicate the positions of isozymes of α-amylase and β-amylase respectively. The α-amylase isoforms are numbered in an order based on their increasing mobility on non-denaturing PAGE. A. Non-denaturing-PAGE of fractions obtained at different steps of purification. The samples after separation by PAGE were stained for protein by silver staining. Lane 1, Dextrin eluate of amylose chromatography; Lane 2, Peak-I of DEAE-cellulose chromatography of dextrin eluate; Lane 3, Peak-II of DEAE-cellulose chromatography of dextrin eluate; Lane 4, Early fractions of Sephadex G-100 chromatography (α-amylase-4), Lane 5, Late fractions of Sephadex G-100 chromatography (α-amylase-3); Lane 6, Peak-I of DEAE-cellulose chromatography of glycogen-enzyme complex. B. SDS-PAGE of fractions obtained at different steps of purification. The samples after separation by SDS-PAGE were stained for protein by Coomassie blue. Lane 1, Dextrin eluate of amylose chromatography; Lane 2, Peak-I of DEAE-cellulose chromatography of dextrin eluate; Lane 3, Peak II of DEAE-cellulose chromatography of dextrin eluate; Lane 4, Early fractions of Sephedex G-100 chromatography (α-amylase-4); Lane 5, Late fractions of Sephadex G-100 chromatography (α-amylase-3); Lane 6, Glycogen-enzyme complex of unbound fraction of amylose chromatography; Lane 7. M_r markers. C. Activity staining of amylases. The crude extract and purified α-amylase-3/4 and β-amylase from 7-day-old endosperm of maize seedlings were subjected to non-denaturing-PAGE and stained for amylase activity. Lane 1, Purified α-amylase-3; Lane 2, purified α-amylase-4; Lane 3, purified β-amylase; Lane 4, crude extract.

Table 1. Purification of amylases from endosperm of 6-day-old maize seedlings

Step	Volume (ml)	Activity (nkat × 10^3)	Protein (mg)	Sp. activity (nkat × mg)	Yield (%)
Crude extract	715	55	775	719	100
$(NH_4)_2SO_4$ (20–65%)	160	437	176	2480	78.5
Amylose column					
a) Unbound	150	158	129	1220	28.4
b) Glycogen precipitate	48	63	4.8	13,200	11.3
c) DEAE Peak-1 (β-amylase)	4.7	33	1.7	19,400	5.9
Amylose column					
a) Dextrin eluate	125	39	25	1560	7
b) DEAE Peak-1 (α-amylase-1,2)	5.2	1.05	8.8	120	0.2
c) DEAE Peak-2 (α-amylase-3,4)	26.5	29	9.6	2980	5.1

(Fig. 1A, lane 4; B, lane 4; C, lane 2), and α-amylase-3 in the late fractions (Fig. 1B, lane 5; C, lane 1). However, the amount of α-amylase-3 was very low, and only a faint protein band could be visualized on PAGE (Fig. 1A, lane 5; B, lane 5; C, lane 1). α-Amylase-1/2 could not be resolved further by gel filtration and was used directly for the analysis of their properties and raising antibodies.

Purified α-amylase-1/2 migrated as independent isoforms on non-denaturing PAGE, but on SDS-PAGe, only a single band of 46 kD was visualized, indicating that both the isoforms possessed similar M_rs. In contrast, α-amylase-3/4 could be resolved into two distinct bands on SDS-PAGE and possessed M_rs of 44.5 and 47.5 k respectively. In another study, amylase isoenzymes from maize endosperm on renaturation after SDS-PAGE showed a major broad band with a M_r of ca 40 k [10]. This band presumably consisted of several closely migrating α-amylase proteins.

Maize β-amylase did not bind to potato amylose and was obtained in the unbound fraction, which also contained some α-amylase activity. Nevertheless, a substantial purification of β-amylase present in the unbound fraction could be achieved by adsorbing it to glycogen, and precipitating the glycogen–enzyme complex using a protocol essentially developed for the purification of α-amylase [17]. Since the unbound fraction also contained some α-amylase activity, particularly that of α-amylase-4, glycogen precipitation also copurified α-amylase-4 activity with β-amylase (Fig. 1B, lane 6). Subsequent chromatography on DEAE-cellulose column rendered β-amylase free of α-amylase-3 activity (Fig. 1A, lane 6; C, lane 3), and purified it substantially. Similarly, in another study in maize, affinity chromatography on cycloheptaamylose-Sepharose 6B column only partially bound α-amylase, and the unbound fraction consisted of both α- and β-amylase [18]. Similarly, abundant β-amylase from pea epicotyl could be purified by glycogen precipitation [19].

In the present study, both Western blotting and *in vivo* labeling of β-amylase from different organs showed an identical M_r of 56 k. However, in another study, a M_r of 65 k was reported for maize β-amylase [20]. In yet another study, the M_r of β-amylase in maize was reported to be 60 k [21]. Recently, β-amylase cDNA clones were obtained from maize seeds and the M_r predicted from the sequence was 54 k [13] which is close to the M_r of 56 k determined in this study.

Immunological characterization

Purified α-amylase-1/2, α-amylase-3/4 and β-amylase proteins were injected in rabbits to raise polyclonal antibodies. α-Amylase-1/2 were antigenically distinct from α-amylase-3/4, and no cross-reactivity was observed between the two groups by immunodiffusion (data not shown) or by Western blotting of the crude extracts (Fig. 2) Similarly, antibodies against β-amylase specifically cross reacted with β-amylase from seeds (Fig. 2).

Time course of amylases increase in maize seed

Extracts from dry seeds homogenized in the presence of 2-mercaptoethanol showed isoforms of α-amylase-3 and -4 and β-amylase, but α-amylase-1/2 could

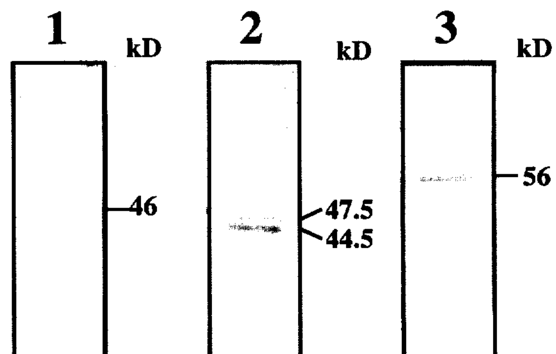

Fig. 2. Western blotting amylases from crude extract of maize seeds. The crude extracts after SDS-PAGE and blotting were probed for the presence of α-amylases-1/2, α-amylase-3/4 and β-amylase using respective polyclonal antibodies. Lane 1, α-amylases-1/2; Lane 2, α-amylase-3/4; Lane 3, β-amylase.

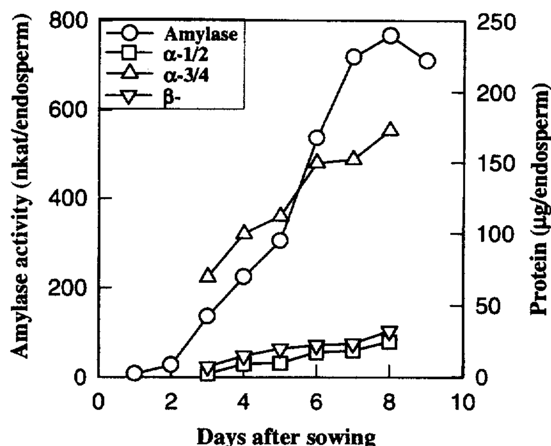

Fig. 3. Time course of amylase activity (Amylase; ○) in the endosperm of maize seedlings. The level of α-amylase-1/2 (α-1/2; □) and α-amylase-3/4 (α-3/4; △) proteins were determined by single radial immunodiffusion and that of β-amylase (β-; ▽) protein by rocket immunoelectrophoresis. The antigen levels of these proteins were below the detectability limit on 0–2 days of germination.

not be detected (data not shown). After sowing the seeds, increase in amylase activity was discernible in the endosperm 2 day onwards, and amylase activity increased linearly up to 8 day (Fig. 3). The immunoquantitation of α-amylase-1/2 and α-amylase-3/4 levels by single radial immunodiffusion revealed that during this period, the levels of the above proteins also increased. During the same period, a significant increase in the level of β-amylase protein was also observed. A comparison of the relative amounts of amylase proteins 7 days after sowing showed that α-amylase-3/4 were the predominant amylases in the endosperm, and their level was 6.2- and 8.4-fold higher than that of β-amylase and α-amylase-1/2 respectively.

Amylase activity increased in the germinating maize seeds, as in other cereals [22, 23], where these enzymes degrade the starchy endosperm to provide energy to the developing seedling till it attains photoautotrophy. The observation that the immunodetectable levels of the various amylase proteins also increase significantly during the period indicates that the increase in amylase activity results either from the *de novo* synthesis of respective amylases (Fig. 3), or that they are solubilized from their insoluble zymogen forms. Since in this study the seed along with the aleurone layer and the scutellum was used for amylase activity assays, and also for the immunoquantitation of the respective proteins, the observed increase in amylase level may be confined only to the aleurone layers, or may have resulted from the release of the enzyme from the scutellum. In view of this, we next monitored the synthesis and secretion of amylases from isolated aleurone layers and scutellum.

Synthesis and secretion of amylases in aleurone layer

The observed increase in the amylase activity in the seed could be due to the synthesis and secretion of amylases from the encircling tissues, such as the scutellum and the aleurone layer. The above possibility was examined by isolating the aleurone layers from the germinating seeds at different time points from sowing, and monitoring the *in vivo* protein synthesis by incubating them in a medium containing ^{35}S-methionine. Immunoprecipitation of α-amylases from tissue homogenates and medium revealed an active synthesis and secretion of α-amylases from the aleurone layer. Synthesis of α-amylase-1/2 was discernible from day 3 onwards, reached a maximum between day 5–6 and then declined. Estimation of α-amylase-1/2 proteins in the incubation medium revealed that the secretion of α-amylase-1/2 followed a profile similar to its synthesis *in vivo* (Fig. 4A). α-Amylase appears as a doublet in the tissue, but in the medium, a single band was observed (Fig. 4A). This could be the result of post-translational processing of the enzyme preceding its secretion into the medium.

Aleurone layers also synthesized α-amylase-3 from day 2 and α-amylase-4 from day 3 onwards till day 8; with a maximum on day 5, but a significant secretion of α-amylase-4 could be detected only during day 5–

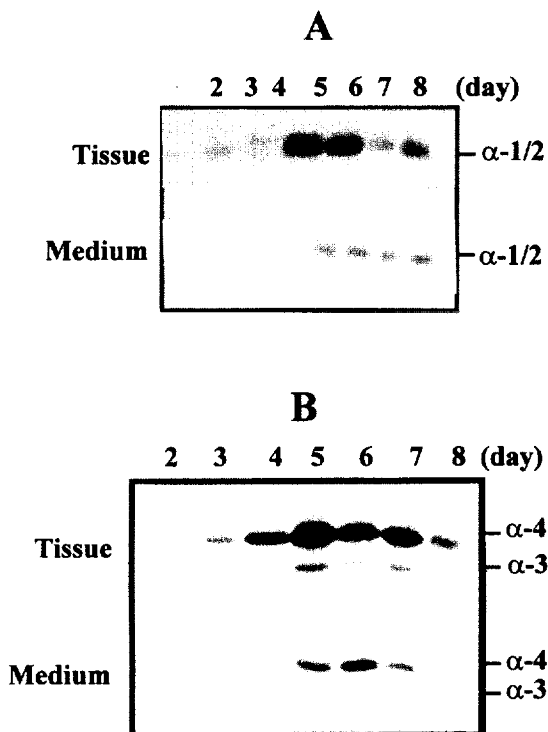

Fig. 4. *In vivo* synthesis and secretion of amylases from aleurone layers at different time points from sowing. At the time points indicated the aleurone layers were isolated and radiolabeled by incubating them for 3 h with ^{35}S-methionine. After homogenization the newly synthesized amylases were immunoprecipitated, subjected to SDS-PAGE and visualized by fluorography. Amylases released in the incubation medium were directly immunoprecipitated and subjected to SDS-PAGE and fluorography as above. A. α-amylase-1/2. B. α-amylase-3/4. The symbol α- on figures indicate the position of the isozymes of α-amylase.

8. In contrast, maximal synthesis of α-amylase-3 was observed during day 5–7 and secretion was detected on day 6–7. In general, both the synthesis and the secretion of α-amylase-4 was higher than α-amylase-3 during this period (Fig. 4B). *In vivo* labeling of aleurone layers showed that it also synthesized β-amylase, and the rate of synthesis was maximal on day 2 followed by a gradual decline (data not shown).

Synthesis and secretion of amylases in scutellum

Amylase activity was also monitored in the scutellum isolated from germinating seeds at different time points. After sowing, amylase activity increased, with maximal increase during day 4–6, and then declined (Fig. 5). Interestingly, on immunoquantitation of α-amylase-1/2 protein, we could detect α-amylase-1/2 protein in the scutellum only up to day 4 from sowing. In contrast, the increase in α-amylase-3/4 protein level followed the profile of α-amylase activity, with a steep increase between day 4–6 followed by a decline (Fig. 5). On *in vivo* labeling of isolated scutellum, immunodetectable labeled α-amylase-1/2 protein was recovered only in the medium, and no radiolabeled α-amylase-1/2 protein could be detected in the tissue (Fig. 6A), indicating that it is rapidly secreted out of the scutellum after synthesis.

In vivo labeling also revealed a clear temporal separation in initiation and peak of α-amylase-3 and α-amylase-4 synthesis (Fig. 6B). α-Amylase-3 appeared on day 2, when its synthesis and secretion were maximal. By day 4 it could not be detected, either in the tissue or in the medium. In comparison, α-amylase-4 synthesis and secretion could be detected from day 3–8 (Fig. 6B). It is apparent from the above observation that the scutellar tissue synthesizes and secretes α-amylase-1/2 and of α-amylase-3 up to day 3 from sowing, and thereafter only α-amylase-4 is synthesized and released from the scutellum.

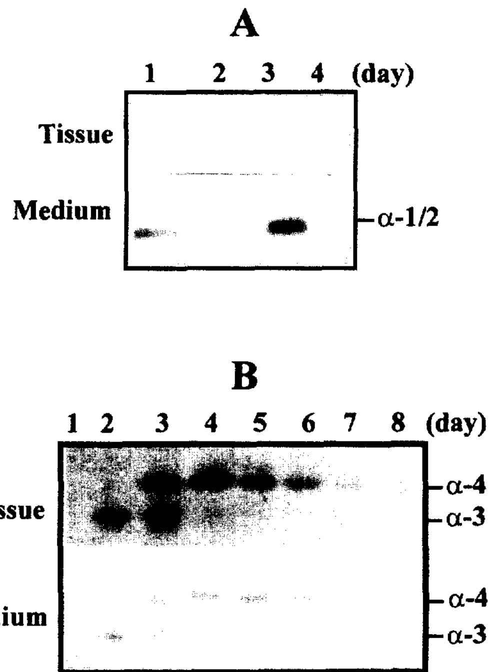

Fig. 6. *In vivo* synthesis and secretion of amylases from scutellum at different time points from sowing. At the time points indicated the scutellums were isolated and were *in vivo* labeled as described in Fig. 2. The amylase polypeptides were immunoprecipitated, subjected to SDS-PAGE and fluorographed. A. α-amylase-1/2. B. α-amylase-3/4. The symbol α- on figures indicate the position of the isozymes of α-amylase.

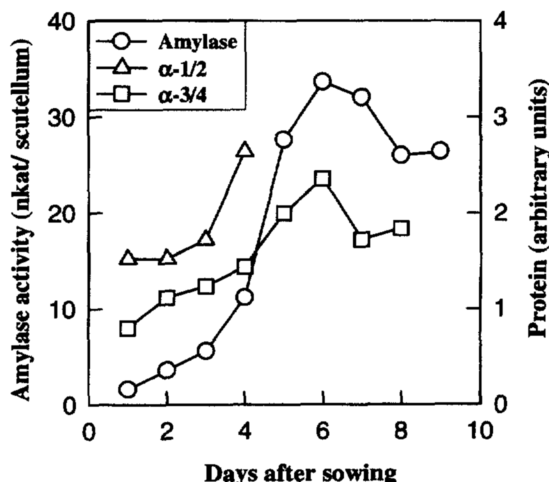

Fig. 5. Time course of amylase activity (amylase; ○) in the scutellum. The level of α-amylase-1/2 (α-1/2; △) and α-amylase-3/4 (α-3/4; □) proteins in scutellum was determined by rocket immunoelectrophoresis.

Effect of Ca^{2+} *on synthesis and secretion of amylases*

In the aleurone layers, Ca^{2+} (10 mM) stimulated the rate of synthesis of α-amylase-1/2, but no increase in the secretion of the newly synthesized α-amylase-1/2 was observed in the medium (Fig. 7A-a). Rather, the presence of 1 mM Ca^{2+} inhibited the secretion of α-amylase-1/2. Ca^{2+} also stimulated the synthesis of α-amylase-4 and α-amylase-3 but no significant effect could be observed on the release of α-amylase into the medium (Fig. 7A-b). In fact, it rather inhibited α-amylase-3 release in the medium. Ca^{2+} also stimulated the synthesis of β-amylase (Fig. 7A-d); however, no significant secretion of β-amylase was observed in the presence of Ca^{2+}. In contrast, Ca^{2+} (1 mM) stimulated the synthesis of α-amylase-4 in the scutellum, but inhibited its secretion into the medium (Fig. 7A-c).

The inclusion of Ca^{2+} ionophore A23187 in the medium abolished the α-amylase-1/2 synthesis at 1 mM, but at 0.01 mM it promoted the secretion of α-amylase-1/2 (Fig. 7B-a). The rate of α-amylase-4 synthesis was not affected by 0.01 mM and 0.1 M concentration of ionophore (Fig. 7B-b). But the syn-

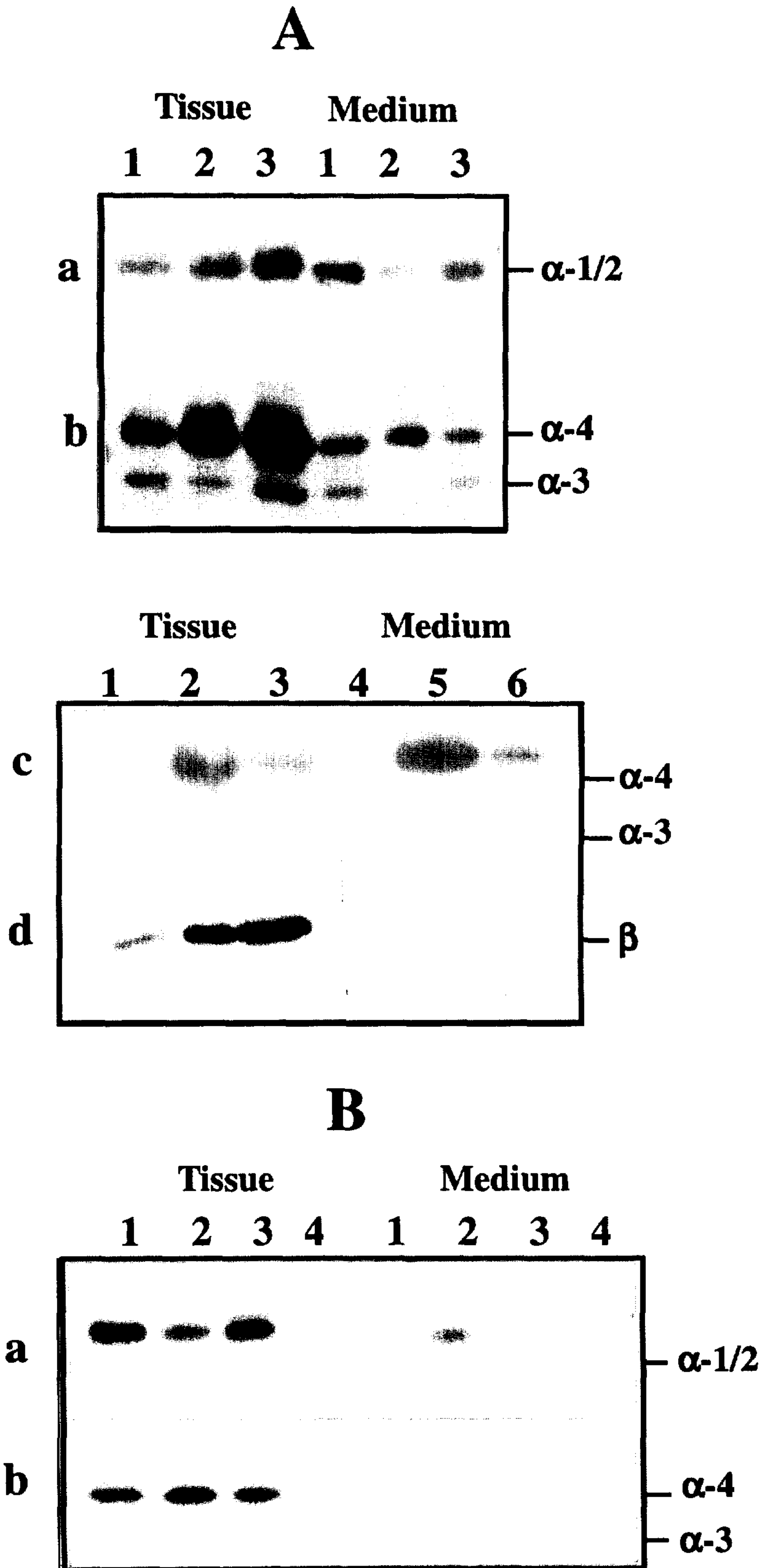

Fig. 7. Effect of Ca^{2+} on the synthesis and secretion of amylases. The aleurone layers and the scutellum isolated from 4-day-old maize seedlings were incubated with varying concentrations of Ca^{2+} (A) or its ionophore (B) and were *in vivo* labeled as described above in Figs 2 and 4. A. Effect of different concentration of Ca^{2+}. a. Aleurone layer, α-amylase-1/2, b. aleurone layer, α-amylase-3/4, c. scutellum, α-amylase-3/4, d. aleurone layer, β-amylase. Lane 1, control; Lane 2, 1 mM Ca^{2+}; Lane, 3, 10 mM Ca^{2+}. B. Effect of different concentrations of Ca^{2+} ionophore on aleurone layers: a. α-amylase-1/2, b. α-amylase-3/4. Lane 1, control; Lane 2, 0.01 mM; Lane 3, 0.1 mM; Lane 4, 1 mM.

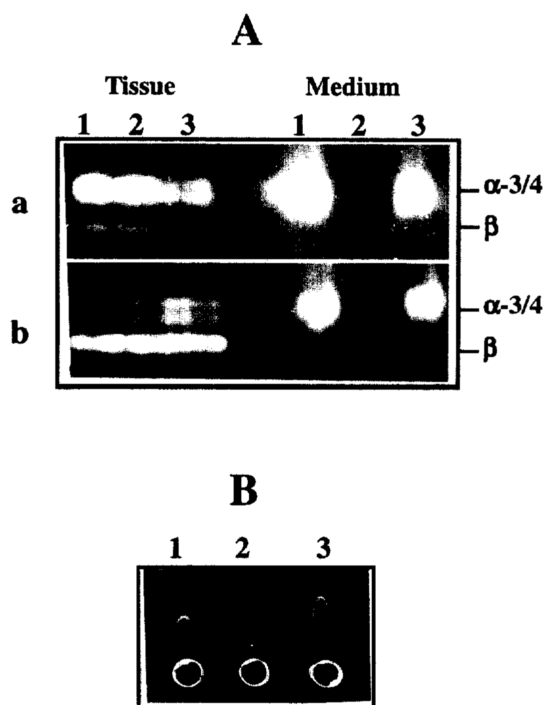

Fig. 8. Effect of EGTA on the synthesis and secretion of amylases. The aleurone layers and scutellum isolated from 4-day-old maize seedlings were incubated with EGTA (1 mM) for 2 h. After non-denaturing-PAGE the amylase isoenzymes were visualized in the tissue and in the medium by staining the gels for amylase activity. Rocket immunoelectrophoresis was conducted using anti-α-amylase-3/4 serum A. a. Aleurone layer, α-amylase-3/4 and β-amylase. Lane 1, control; lane 2, +EGTA; lane 3, + $CaCl_2$ (10 mM). b. Scutellum, α-amylase-3/4 and β-amylase. Lane 1, control; lane 2, +EGTA; lane 3, +$CaCl_2$ (1 mM). B. Rocket immunoelectrophoresis of α-amylase-3/4 released into the medium from aleurone layer. Lane 1, control; Lane 2, +EGTA; Lane 3, +$CaCl_2$ (10 mM).

thesis and secretion of all the α-amylase isoforms were inhibited by treatment with 1 mM Ca^{2+} ionophore.

Incubation of aleurone layers with 1 mM EGTA completely abolished amylase activity in the medium (Fig. 8A-a, lane 2). Since EGTA can also possibly inactivate α-amylases by chelating the calcium present in the enzyme molecules, the presence of α-amylase apoproteins in the medium was ascertained by rocket immunoelectrophoresis. Figure 8B shows that EGTA completely inhibits the secretion of amylase protein. In the presence of EGTA, no immunodetectable α-amylase-3/4 protein was observed in the medium, while the same could be detected in the tissue. Amylase activity in the scutellum also was stimulated by exogenous Ca^{2+} and inhibited by the chelation of Ca^{2+} by EGTA (Fig. 8A-b). In the scutellum also, EGTA inhibited the release of α-amylases in the medium (Fig. 8A-b, lane 2).

In 6-day-old maize seeds, the release of β-amylase activity from the aleurone layer and the scutellum in the medium could be stimulated by calcium [24]. In contrast, we observed that in 4-day-old maize seeds, though Ca^{2+} stimulated the synthesis of β-amylase in the aleurone layers, it could not be detected in the medium (Fig. 8A-a). Also, we could not detect any release of β-amylase from the scutellum into the medium, though it possessed high β-amylase activity (Fig. 8A-b). In an earlier study, only a minor fraction of the β-amylase (ca 6%) was reported to have been released from the aleurone layer of 6-day-old seeds into the medium [24]. However, our results and those of Lauriere *et al.* [24] are in contrast to the recent study of Wang *et al.* [13], where they could not detect any β-amylase mRNA in the scutellum of maize. It is likely that the β-amylase observed in the scutellum in this study was inherited by the seed from the time of maturation, as we have also detected β-amylase in the extracts of dry seeds (data not shown).

In vivo labeling of the aleurone layers and the scutellum and immunoprecipitation of the labeled α-amylases clearly showed that during germination, both the organs synthesize amylases *de novo* and release them into the medium. Although a hormonal induction of α-amylase synthesis has not been observed for maize [8, 9], α-amylase synthesis in maize is modulated by the availability of calcium as in barley [7]. Similar to the previous report [12], the secretion of α-amylase was calcium independent in maize. However, chelation of calcium by EGTA significantly lowered the secretion of maize α-amylases into the medium. Since the Ca^{2+} ionophore A23187 stimulated the synthesis of α-amylase-1/2 and α-amylase-4 in the aleurone layer in low concentrations, it can be assumed that this above effect is caused by its ability to move Ca^{2+} across the membranes [25].

There has been a considerable debate on the relative importance of the scutellum and the aleurone layer in initiating starch mobilization during cereal seed germination [22, 26]. The results obtained in this study demonstrate the role of the scutellar tissue in initiating starch mobilization during the initial period of maize seed germination. While both the scutellum and the aleurone layer synthesize and secrete amylases, the synthesis and secretion pattern of α-amylases in the scutellum differ from that of the aleurone. The synthesis of α-amylases starts first in the scutellum; and most of the newly synthesized α-amylases are quickly secreted into the medium. Within 24 h of inhibition of the seed, the synthesis and secretion of amylase-1/2 are evident in the scutellum, followed by the synthesis and secretion of α-amylase-3 and α-amylase-4 on day 2 and 3 respectively.

In contrast, in the aleurone layers, the synthesis and secretion of α-amylases start later than the scutellum; however, it persists for a longer period. While the ability of the scutellum to secrete α-amylases is lost after day 4, in the aleurone layer, α-amylase synthesis and secretion peak between day 5–7 and gradually decline thereafter. Evidently in maize also, as to barley [20] and rice [21, 22], the scutellum plays an important role in initiating the mobilization of the starchy endosperm.

EXPERIMENTAL

Plant material

Maize (*Zea mays* L., cv. Ganga-5 Hybrid) seeds, purchased from Andhra Pradesh State Seed Corporation, Hyderabad were used in this study. After soaking for 24 h in H_2O at 25°, the seeds were sown on 4 layers of moist germination paper in plastic trays, and the seedlings were grown at $25 \pm 1°$ for 6 days. After 6 days, the seedlings were excised from the seeds and the de-embryonated kernels were used for enzyme purification.

At appropriate time intervals, the seeds were dissected into different parts after removing the shoot and the root. The endosperm inclusive of the aleurone layer was obtained by removing the seed coat and the scutellum. The scutellum was excised from the kernel and the embryo was removed. Care was taken to avoid aleurone layer contamination in the scutellar tissue. Scutellum free kernels were used for the isolation of the aleurone layers. It was possible to detach the aleurone layers from the other tissues only after day 2 after sowing. The seed coat was carefully removed and the aleurone layers were peeled from the endosperm with fine-tipped tweezers. The isolated tissues were immediately placed in a buffer (10 mM Tris-HCl, pH 7.4) to avoid dehydration, and were washed with the above buffer to remove adhering starch.

Amylase assay

Amylase activity was assayed in a reaction mixture containing in a final vol. of 4 ml, 100 mM NaOAc buffer pH 4.6, 5 mM $CaCl_2$, 4 mM NaCl, 8 mg/ml amylose (potato, Sigma) 1.5 mM NaF and appropriately diluted homogenate at 30°. At different intervals, 0.5 ml aliquots were withdrawn and mixed with 0.5 ml [3–5, dinitrosalicylic acid (DNS) reagent, and the increase in reducing sugars was determined by measuring A_{540} using maltose as the standard [30].

α-Amylase assay was performed after inactivation of β-amylase present in the crude extract by heating equal vols of the crude extract and 20 mM $CaCl_2$ soln in a H_2O bath at 70° for 10 min. The assay mixture for α-amylase in a final vol of 4 ml consisted of 100 mM NaOAc pH 5.2, 1 mM NaF and 4 mg/ml β-limit dextrin. To initiate the assay, 0.5 ml of crude enzyme extract was added to the reaction mixture. α-Amylase activity was assayed as described earlier for total amylase. After assaying total amylase activity and α-amylase activity, β-amylase activity was calculated by subtracting the value of α-amylase activity from that of the total amylase activity. The amount of protein in the extracts was estimated by using the procedure described in Ref. [31]. The enzyme activity was expressed as katals (IUB nomenclature), one katal being equivalent to one mole of maltose generated per s during the enzyme reaction.

Amylase purification

De-embryonated maize kernels (500 g) were homogenized in 500 ml of homogenization buffer [100 mM NaOAc, pH 4.6; 5 mM $CaCl_2$; 4 mM NaCl, 4 mM phenylmethylsulfonyl fluoride (PMSF); 80 mM 2-mercaptoethanol] in a Wareing blender and the homogenate was centrifuged at 3000 *g* for 5 min. The pellet was rehomogenized in 300 ml of homogenization buffer, and after centrifugation, the supernatants were pooled and centrifuged at 15,000 *g* for 1 h. The resulting supernatant was subjected to $(NH_4)_2SO_4$ fractionation (20–65%) and the ppt obtained after centrifugation at 15,000 *g* for 30 min was resuspended in 160 ml of homogenization buffer and dialysed against the same buffer (36 h) with several changes.

The dialysate was clarified by centrifugation (10,000 *g*, 10 min) and loaded onto a potato amylose (Sigma) column (15 cm × 1.2 cm), pre-equilibrated with homogenization buffer. The column was extensively washed with homogenization buffer and the unbound amylase fraction was collected for glycogen precipitation (see below). Bound amylases were eluted at 25° by application of a soln of saturated white dextrin (Loba Chemie, product resulting from controlled hydrolysis of wet starch) dissolved in homogenization buffer. Fractions containing amylase activity were pooled (125 ml) and dialysed against ion exchange buffer containing 50 mM Tris-HCl, pH 8.5; 5 mM $CaCl_2$; 2 mM PMSF; 5 mM 2-mercaptoethanol for 24 h with 3 changes. The dialysate was loaded onto a DEAE-cellulose (Sigma) column (5 cm × 1.5 cm), which was pre-equilibrated with the above ion exchange buffer. After an extensive washing with the above buffer, the bound proteins were eluted by a linear gradient of 0–0.5 M NaCl at a flow rate of 8 ml/h. The fractions constituting two peaks of amylase activity were separately pooled and dialysed against homogenization buffer. Fractions constituting peak I of amylase was pooled, dialysed and used for further study. Peak II of amylolytic activity was further fractionated on Sephadex G-100 column (180 cm length × 2.5 cm diam) at a flow rate of 5 ml/h pre-equilibrated with homogenization buffer and the two amylase peaks as judged by SDS-PAGE were separately pooled and used for further study.

Unbound amylase from amylose affinity chromatography was subjected to glycogen precipitation as described in Ref. [17]. The unbound fraction was first made to 40% EtOH (v/v) and after centrifugation (10,000 *g*, 20 min), the supernatant was made to 20 mM Na-Pi buffer, pH 8). Thereafter, glycogen (Sigma, type II from Oyster) soln [2% (w/v)] was added to the supernatant (10 mg glycogen/750 nkat amylase) and EtOH was readjusted to 40%. The enzyme-glycogen complex was collected after 15 min by centrifugation at 5000 *g* and was washed 3 times with 10 mM Na-Pi buffer, pH 8 containing 40% EtOH. The complex was dispersed in homogenization buffer and incubated at 30° for 3 h in order to allow the amylase to degrade

the residual glycogen. Thereafter, the supernatant obtained after centrifugation at 10,000 g for 5 min, was dialysed against 50 mM Tris-HCl, pH 7; containing 5 mM 2-mercaptoethanol. The dialysate was subjected to ion exchange chromatography on a DEAE-cellulose column (5 cm × 1.5 cm) as described above using 50 mM Tris-HCl buffer pH 7. Two amylase peaks, peak I containing β-amylase and peak II containing α-amylase were collected.

Electrophoresis

PAGE under non-denaturing conditions was conducted in 10% (w/v) slab gels in a custom made gel apparatus according to the method of Ref. [32]. Six nkat of amylase activity in 20% (v/v) glycerol was loaded in each lane. Amylase bands were visualized by incubating the gels in a soln containing 2% amylose (w/v) dissolved in 0.1 M NaOAc buffer pH 4.6 containing 10 mM $CaCl_2$ for 3 h at 37°. The gel was rinsed in H_2O and stained with 0.2 M HCl containing 5.7 mM I_2 and 43.3 mM KI. Amylase bands appeared as clear areas against a deep blue background [33]. SDS-PAGE was performed using the according to Ref. [34] in 10% acrylamide gel, and stained using Coomassie blue (Fig. 1B). The non-denaturing gel of Fig. 1A was Ag stained. Isoelectrofocusing was done in glass tubes (10 cm × 2.5 mm i.d.) [35]. A gel run without loading sample was cut into 0.5 cm sections, and incubated in a soln of freshly prepared 9.2 M urea for 15 min. The pH of the resulting solns were measured and a standard graph of pH against the gel length was plotted. The isoelectric point was estimated using this standard graph. The M_rs of the purified amylases were determined using the following protein standards; α-alctalbumin (14.2 k), trypsin inhibitor (20.1 k), trypsinogen (24 k), carbonic anhydrase (29 k), albumin, egg (45 k), albumin, bovine (66 k).

In vivo *labeling*

For *in vivo* labeling, the respective tissues excised from the seeds were incubated in 100 μl of sterile 10 mM Tris-HCl, pH 7.4; 10 mM $CaCl_2$ containing 30 μCi of ^{35}S-methionine (> 1000 Ci/mmol, Amersham) for 3 h at 25° with constant shaking in 1.5 ml Eppendorf tubes. Thereafter, the tissues were washed 3 times with 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, and homogenized in 1 ml of the above buffer supplemented with 2% (w/v) polyvinylpolypyrrolidone. The homogenate was centrifuged (20,000 g) in a microfuge for 10 min and the clear supernatant was made up to 0.8% SDS (w/v) and boiled for 4 min. The supernatant was subjected to immunoprecipitation, SDS-PAGE and fluorography using the procedure described below. A 10 μl aliquot was withdrawn from the supernatant for determination of total incorporation of radiolabeled methionine into protein. The estimation of radioactivity incorporation in protein was done by TCA precipitation as described earlier [36]. While α-amylase-1/2 on *in vivo* labeling and SDS-PAGE migrated as a single broad brand of 46 kD, α-amylase-3 and α-amylase-4 could be resolved as proteins of 44.5 and 47.5 kD respectively. The numbering of α-amylase on radiolabeled gels refers to the corresponding isoform detected on native-PAGE.

The rate of secretion of amylase into the medium during the incubation period was estimated by subjecting the medium to immunoprecipitation. After removing the tissue, the vol of the medium was made up to 0.5 ml with homogenization buffer and a 10 μl aliquot was withdrawn for estimating TCA precipitable radioactivity.

After ascertaining the total incorporation of radiolabel in the proteins, aliquots containing equal radioactivity were drawn from the homogenate/medium for immunoprecipitation. The aliquot vol was made to 0.5 ml with homogenization buffer and an equal vol. of dilution buffer [50 mM Tris-HCl, pH 7.4; 6 mM EDTA; 0.3 M NaCl; 1% Triton X-100 (w/v)] was added. It was subjected to immunoprecipitation, electrophoresis and fluorography as described below.

The *in vivo* labeled products were immunoprecipitated by the addition of 15 μl of the respective antisera to the diluted translation mixture or extract. The mixture was incubated at 37° for 1 h followed by overnight incubation at 4°. Thereafter, immunoprecipitate was adsorbed on *Staphylococcus aureus* cells (Cowan I strain, 32) (30 μl, 10%) by continuous shaking at 25° for 2 h. The cells were collected by centrifugation in a microfuge for 30 s and the immunoprecipitated amylases were visualized after SDS-PAGE by fluorography [36].

Effect of Ca^{2+} *on synthesis and secretion*

After excision, the tissues were first incubated for 2 h in 10 mM $CaCl_2$ or 1 mM EGTA in 50 mM Tris-HCl, pH 7.4 containing 1 mg/ml of streptomycin and chloramphenicol and then transferred to fresh buffer for another 3 h. At the end of the incubation period, the tissues and also the media were separately assayed for amylase activity by non-denaturing PAGE [32] and rocket immunoelectrophoresis [37].

Effect of Ca^{2+} on the synthesis and secretion of amylases was assayed by incubating the tissues in 50 mM Tris-HCl, pH 7.4 with or without Ca^{2+}, containing 30 μCi of ^{35}S-methionine with continuous shaking. At the end of 3 h, the synthesis and secretion of amylases were determined by immunoprecipitation as described above. The effect of Ca^{2+} ionophore A23187 (Sigma) from 0.01 to 1 mM was similarly assayed as described above except that the tissues were also preincubated in a buffer containing the above ionophore but without ^{35}S-methionine for 2 h.

Acknowledgements—Supported in part by University Grants Commission, New Delhi grant [(3-42/91(SR-II)] and Council of Scientific and Industrial Research, New Delhi grant [38(0887)/95/EMR-II] to R.S.

K.V.S. and R.D. are recipients of senior research fellowships from the University Grants Commission, New Delhi.

REFERENCES

1. Fincher, G. B., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1989, **40**, 305.
2. Akazawa, T., Mitsui, T. and Hayashi, M., In *The Biochemistry of Plants*. Vol. 14, ed. P. K. Stumpf and E. E. Conn. Academic Press, New York, 1988, p. 465.
3. Nolan, R. C. and Ho, T.-H. D., *Plant Physiology*, 1988, **88**, 588.
4. Deikman, J. and Jones, R. L., *Plant Physiology*, 1985, **78**, 192.
5. Muthukrishnan, S., Gill, B. S., Swegle, M. and Chandra, G. R., *Journal of Biological Chemistry*, 1984, **259**, 13637.
6. Jones, R. L. and Carbonell, J., *Plant Physiology*, 1984, **76**, 213.
7. Nelson, O. and Pan, D., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1995, **46**, 475.
8. Rood, S. B., Oaks, A., Bilan, P. J. and Pharis, R. P., *Plant Physiology*, 1989, **89** Supplement, 170.
9. Goldstein, L. D. and Jennings, P. H., *Plant Physiology*, 1975, **55**, 893.
10. Segundo, B. S., Casacubarta, J. M. and Puigdomenech, P., *Plant Science*, 1990, **69**, 167.
11. Warner, D. A. and Knutson, C. A., *Plant Science*, 1991, **78**, 143.
12. Thevenot, C., Lauriere, C., Mayer, C., Simond-Cote, E. and Daussant, J., *Journal of Plant Physiology*, 1992, **140**, 61.
13. Wang, S. M., Lue, W. L., Wu, S. Y., Huang, H. W. and Chen, J., *Plant Physiology*, 1997, **113**, 403.
14. Okamoto, K. and Akazawa, T., *Agricultural and Biological Chemistry*, 1978, **42**, 1379.
15. Lecommandeur, D., Sirou, Y. and Laurière, C., *Archives of Biochemistry and Biophysics*, 1990, **278**, 245.
16. Silvanovich, M. P. and Hill, R. D., *Analytical Biochemistry*, 1976, **73**, 430.
17. Schramm, M. and Loyter, A., *Methods in Enzymology*, 1966, **8**, 533.
18. Warner, D. A., Grove, M. J. and Knutson, C. A., *Cereal Chemistry*, 1991, **68**, 383.
19. Lizotte, R. A., Henson, C. A. and Duke, S. H., *Plant Physiology*, 1990, **92**, 615.
20. Doyen, C. and Laurière, C., *Phytochemistry*, 1992, **31**, 3697.
21. Wang, S. M., Wu, S. Y. and Chen, J., *Botanical Bulletin of Academia Sinica (Taipei)*, 1992, **33**, 359.
22. Macgregor, A. W., Macdougall, F. H., Mayer, C. and Daussant, J., *Plant Physiology*, 1984, **75**, 203.
23. Filner, P. and Varner, J. E., *Proceedings of National Academy of Science U.S.A.*, 1967, **58**, 1520.
24. Lauriere, C., Doyen, C., Thevenot, C. and Daussant, J., *Plant Physiology*, 1992, **100**, 887.
25. Gardiner, J. D., Walker, M. D. and Rottman, A. J., *American Journal of Physiology*, 1980, **238**, 9458.
26. Gram, N. H., *Carlsberg Research Communication*, 1982, **47**, 143.
27. Ranki, H. and Sopanen, T., *Plant Physiology*, 1984, **75**, 710.
28. Okamoto, K. and Akazawa, T., *Plant Physiology*, 1979, **63**, 336.
29. Thevenot, C., Simond-Cote, E. and Daussant, J., *Physiologia Plantarum*, 1991, **82**, 249.
30. Bernfeld, P., *Methods in Enzymology*, 1955, **1**, 149.
31. Lowry, O. H., Rosenbrough, N. J., Farr, A. H. and Randall, G. J., *Journal of Biological Chemistry*, 1951, **193**, 256.
32. Davis, B., *Annals of New York Academy of Science*, 1964, **121**, 404.
33. Subbaramaiah, K. and Sharma, R., *Journal of Biochemical and Biophysical Methods*, 1985, **10**, 315.
34. Laemmli, U. K., *Nature*, 1970, **227**, 680.
35. Jackson, J. and Hunt, T., *Methods in Enzymology*, 1983, **96**, 50.
36. Subbaramaiah, K. and Sharma, R., *Plant Physiology*, 1989, **89**, 860.
37. Laurell, C. B. and McKay, E. J., *Methods in Enzymology*, 1981, **73**, 339.